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<b>(21) International Application Number:</b> <b>PCT/US90/05451</b> <b>(22) International Filing Date:</b> <b>25 September 1990 (25.09.90)</b>  <b>(30) Priority data:</b> 412,303                      25 September 1989 (25.09.89) <b>US</b> 581,713                      13 September 1990 (13.09.90) <b>US</b>  <b>(71) Applicants:</b> <b>GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US). CRC TECHNOLOGY LTD. [GB/GB]; 2 Carlton House Terrace, London SW1Y 5AR (GB).</b>  <b>(72) Inventors:</b> <b>PRAGNELL, Ian, B. ; 14 Belmont Crescent, Glasgow G12 (GB). DONALDSON, Debra, D. ; 40 1/2 Roberts Road, Cambridge, MA 02138 (US). GRAHAM, Gerald, J. ; 31 Edgemont Street, Glasgow G41 3EH (GB). WONG, Gordon, G. ; 40 Jamaica Way, Apt. 10, Jamaica Plain, MA 02130 (US).</b>		<b>(74) Agents:</b> <b>KAPINOS, Ellen, J. et al.; Genetics Institute, Inc., 87 Cambridge Park Drive, Cambridge, MA 02140 (US).</b>  <b>(81) Designated States:</b> <b>AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</b>  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> <b>METHOD FOR INHIBITING GROWTH OF STEM CELLS</b>  <b>(57) Abstract</b> <p>The present invention discloses a method for protecting cycling or dividing stem cells from the cytotoxic effects of chemotherapeutic agents and radiation by administering prior to exposure to these agents, an effective amount of Stem Cell Inhibitory Factor as well as a composition useful therefor.</p>		

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## METHOD FOR INHIBITING GROWTH OF STEM CELLS

The present invention relates generally to the treatment of human or animal subjects anticipating exposure to chemotherapeutic agents, radiation exposure, or other agents which irreparably damage cycling stem cells and a stem cell inhibitory composition useful therefore.

Cross-Reference to Related Application

This application is a continuation-in-part of U. S. Patent Application, Serial No. 07/412,303, filed September 25, 1989.

Background of the Invention

In the treatment of subjects with cancer, conventional chemotherapy involves the application of one or more cell cycle specific cytotoxic agents, which have the adverse effect of killing, or irreparably damaging,

both normal and cancer cells undergoing division. The goal of such chemotherapeutic agents, e.g., cytosine arabinoside (ara-C), is to destroy or disable the dividing cancer cells, preventing further cancer cell growth. A presently unavoidable effect of chemotherapy, however, is the destruction of other normal dividing cells, particularly the stem cells of the hematopoietic system and the epithelial stem cells which line the scalp and gut. Stem cell damage caused by the administration of chemotherapy, result in the usual side effects thereof, such as hair loss, stomach and intestinal damage, skin damage, myelosuppression, anemia, reduced immune function or response and the resulting increased sensitivity to infection.

A characteristic property of stem cells is their quiescent nature within the cell cycle. When there is 'insult' to the bone marrow, such as by drug treatment, radiation, severe blood loss, inflammatory reaction, or infection, the stem cell responds by a feedback mechanism to begin cycling to replenish the more mature progenitor cells, which in turn, differentiate into the required mature cells of the hematopoietic, immune or epithelial systems.

Since the hematopoietic stem cells are necessary for the development of all of the mature cells of the hematopoietic and immune systems, their survival is essential in order to reestablish in the subject

treated with chemotherapy, a fully functional host defense system. Similarly, survival of epithelial stem cells is necessary for repair of the epithelial linings of organs, including the skin. Because high doses of cycle specific chemotherapeutic drugs, such as ara-C, effectively kill hematopoietic stem cells and stem cells of epithelial tissues, patients exposed to such agents suffer from serious side effects and are frequently at risk of serious infections.

Similar destruction of stem cells occurs upon exposure to a variety of dosages of radiation, whether the radiation is used for therapeutic purposes or is the result of accidental or unavoidable exposure to radiation, e.g., incident to a clean-up of, or presence at the location of, a nuclear accident at a power plant, or on a nuclear submarine. Subjects exposed to radiation also experience the destruction of their hematopoietic stem cells, and consequent failure or serious damage to the hematopoietic and immune systems. Radiation also kills dividing epithelial cells with consequent damage to many epithelial tissues.

While agents, such as the colony stimulating factors, e.g., M-CSF, CSF-1, GM-CSF, and others, are presently being employed to stimulate the development of certain hematopoietic cell lineages in subjects exposed to chemotherapy or radiation, such agents are not believed to be capable of restoring the hematopoietic

system if an insufficient quantity of stem cells is present in the subject after such exposure.

Thus, there remains a need in the art for other therapeutic agents capable of protecting stem cells from the damaging effects of chemotherapy or radiation.

#### Summary of the Invention

The present invention addresses this need in the art by providing a method of treating a subject anticipating exposure to an agent capable of killing dividing or cycling stem cells by administering to that subject an effective amount of a stem cell inhibitory composition. The stem cells protected by this method may be hematopoietic stem cells ordinarily present and dividing in the bone marrow. Alternatively, stem cells may be epithelial, located e.g., in the intestines or scalp or other areas of the body. The method of this invention may be desirably employed on humans, although animal treatment is also encompassed by this method.

The stem cell inhibitory composition useful in this method comprises an effective amount of a polypeptide or fragment of a protein referred to herein as Stem Cell Inhibitor Factor (SCIF) and described with specificity below.

In another aspect, the invention provides a method for protecting and restoring the hematopoietic, myeloid and immune systems of a patient undergoing chemotherapy, which includes administering to the patient an effective amount of SCIF. SCIF may be administered prior to the chemotherapy. Alternatively the SCIF may be administered during chemotherapy. Another alternative is administering the SCIF for a period after the chemotherapeutic regimen. Following chemotherapy, the patient may be treated with therapeutic factors, such as colony stimulating factors or other lymphokines that stimulate the stem cells to divide and further stimulate the production of more mature cells of the hematopoietic lineages.

In still a further aspect, the present invention involves a method for adjunctively treating any cancer, including those characterized by solid tumors, by administering to a patient having cancer an effective amount of SCIF to protect the hematopoietic stem cells of the bone marrow, thereby allowing greater dosages of chemotherapeutics or radiation to be employed for treatment of the cancer.

Yet another aspect of the present invention involves the treatment of leukemia comprising treating bone marrow cells having proliferating leukemia cells therein with an effective amount of SCIF to inhibit proliferation of normal stem cells, and treating the bone marrow with a cytotoxic agent to destroy leukemia cells. This method may be enhanced by the follow-up treatment of the bone marrow with other agents that stimulate its proliferation, e.g., lymphokines. This method may be performed in vivo. Alternatively, this method may be performed ex vivo and the resulting marrow purged of leukemia cells by the chemotherapeutic agent. The marrow may then be reinjected into the patient.

In still a further aspect, the method involves treating a subject having any disorder caused by proliferating stem cells. Such a disorder, such as psoriasis, may be treated by administering to the subject an effective amount of SCIF to partially or wholly inhibit proliferation of the stem cell in question.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of preferred embodiments thereof.

Detailed Description of the Invention

The present invention provides a method for reversibly protecting stem cells from damage from a cytotoxic agent capable of killing dividing stem cells.

5 The method involves administering to a subject anticipating exposure to such an agent an effective amount of a Stem Cell Inhibitory Factor (SCIF). The method also may involve extending the SCIF treatment throughout the cytotoxic treatment to enhance the stem  
10 cell protective effect.

1 SCIF can reversibly inhibit division of a variety of stem cells in the human body. Specifically SCIF is effective in temporarily inhibiting cell division of hematopoietic stem cells. Additionally, SCIF also  
15 acts to inhibit cycling or dividing epithelial stem cells, located throughout the body. Other stem cell populations on which SCIF may exercise a reversibly inhibiting activity include male and female germinal cells. SCIF may then be employed to protect a male or  
20 female patient from post-chemotherapeutic germinal aplasia. Additionally, because chemotherapeutic agents commonly induce alopecia and mucositis, SCIF may be used to reversibly protect hair follicles and oro-  
gastrointestinal epithelial stem cells from adverse side  
25 effects of chemotherapy or radiation.

Thus the method of this invention may be employed in alleviating the undesirable side effects of chemotherapy on the patient's hematopoietic, myeloid and immune systems by protecting stem cells from damage from a chemotherapeutic agent, such as a cytotoxic drug or radiation dosage normally used to destroy cancer cells. Such an application of SCIF may also serve to protect epithelial stem cells during chemotherapy. SCIF may be administered to the patient in a dosage sufficient to inhibit stem cell division for a time sufficient to allow the action of the chemotherapeutic agent. After the chemotherapeutic agent has performed its function, the stem cells inhibited by SCIF will without further treatment, revert to dividing cells. If it is desired to enhance the reversion of hematopoietic stem cells, the patient can receive doses of hematopoietic growth factors or cytokines used to stimulate the growth and development of hematopoietic cells.

The majority of chemotherapeutic agents used for cancer chemotherapy have a relatively short in vivo half-life, usually less than 24 hours. This inhibitory effect of SCIF according to the present invention is maintained for at least the major proportion of the effective time during which the chemotherapeutic agent is active in vivo. For those cytotoxic agents which have

prolonged half-lives (e.g., greater than 24 hours), it is expected that more prolonged treatment with SCIF would be required. The normal physiological mechanisms within the subject would limit the effective duration of activity of SCIF in relation to cycling stem cells.

Additionally, the method has utility in providing a subject protection against other exposure to radiation, which damages the bone marrow cells of a subject. An individual may be administered SCIF where unintentional or accidental exposure to dangerous levels of radiation are anticipated. For example, such individuals anticipating entering sites of nuclear fall-out, persons responsible for examining and cleaning nuclear power plants after a leak of dangerous levels of radiation or nuclear submarines and the like may be treated by this method to inhibit replicating stem cell division in case of short term radiation exposure. SCIF administration during, as well as before radiation exposure may provide enhanced protection.

Additionally, SCIF may be employed in this method as an adjunctive treatment with chemotherapy to treat any cancers. Because the bone marrow is the limiting organ in determining the amount of radiation or dosage of cytotoxic drug that can be applied to a patient, SCIF may be used to protect the bone marrow hematopoietic cells

from radiation or chemotherapy, thereby allowing greater amounts of radiation or drugs to be applied to treat any cancer, normally amenable to either radiation or chemotherapeutic treatment. Since the myelotoxicity of the cytotoxic drugs is also limiting on their dosage during chemotherapy, the administration of SCIF to the patient is likely to enable increased drug dosages to be given to the patient without the serious side effects that would normally accompany such increased dosages.

The method may also be employed to treat solid tumors by inhibiting during chemotherapeutic treatment, the division of epithelial stem cells.

SCIF may also be employed in a method for preparing autologous bone marrow for transplantation. The marrow can be treated ex vivo with an effective amount of SCIF to inhibit stem cell division and then purged of cancerous cells by administering an effective amount of a chemotherapeutic agent or radiation. Marrow thus treated may be reinjected into the autologous donor. Optionally the patient may then be treated with an agent known to stimulate hematopoiesis.

SCIF may also be employed in the method of this invention as an adjunctive therapy in the treatment of leukemia. For example, where the leukemic cells do not respond to SCIF, the leukemic bone marrow cells may be

treated ex vivo, with SCIF. The proliferation of normal stem cells is prevented by administration of SCIF. Thus, during the time that the proliferating leukemic cells are treated with a cytotoxic agent, a quantity of normal stem cells are protected from damage. Additionally, a stimulatory cytokine, such as IL-3 or GM-CSF, may be administered to induce cycling in the leukemic cells during drug or radiation treatment while the normal stem cells are protected with SCIF.

For ex vivo use, the bone marrow may be treated with SCIF to inhibit stem cell division, thereby protecting stem cells from destruction by the subsequent application to the marrow or to the source of any other cancer, of chemotherapy designed to destroy cycling cells, e.g. leukemic cells. The resulting purged marrow may be reinjected into the patient, wherein the stem cells will begin to divide normally. To enhance cell division of the hematopoietic stem cells, the lymphokines and cytokines above-identified may also be administered. The same process may be accomplished by administering SCIF to the patient in vivo.

The method of this invention may also be employed to treat disorders related to hyperproliferative stem cells. For example, psoriasis is a disorder caused by hyperproliferating epithelial cells of the skin and is

sometimes treated with cytotoxic drugs. Similarly, a condition of in situ dysplasia of cervical epithelium is recognized which will proceed to cervical cancer if left untreated. Both of these transitional states may be  
5 treated prophylactically with SCIF. Other pre-neoplastic lesions in which stem cell proliferation is involved may also be amenable to effective amounts of SCIF employed to inhibit wholly or partially the proliferation of the stem cells. For these uses, topical or transdermal  
10 compositions containing SCIF may be employed, as well as other parenteral means of administering SCIF.

The SCIF polypeptides may also be used in another method of this invention. Antibodies, monoclonal or polyclonal, may be developed by standard techniques to  
15 the SCIF polypeptides. These antibodies or SCIF polypeptides may be labelled with detectable labels of which many types are known in the art. The labeled SCIF or anti-SCIF antibodies may then be employed as stem cell markers to identify and isolate stem cells by  
20 administering to a patient directly for diagnostic purposes. Alternatively these labeled polypeptides or antibodies may be employed ex vivo to identify stem cells in a bone marrow preparation, to enable their removal prior to purging techniques. In the same manner such

labeled polypeptides or antibodies may be employed to isolate and identify epithelial, or other stem cells.

The inventors herein have discovered that SCIF, employed in the following methods and examples, is the murine homologue of protein factors previously identified. K. Obaru et al, J. Biochem., 99:885-894 (1986) identified the amino acid and DNA sequence of a gene found to be inducible by tumor promoters such as phorbol ester in human tonsillar lymphocytes. The authors predicted that this gene produced a protein which may signal proliferation of T cells. More recently, P. F. Zipfel et al, J. Immunol., 142:1582-1590 (1989) reported that the same protein was produced by mitogenic activation of human T cells and suggested that it may function as a lymphokine or cytokine.

The present method thus desirably employs the human SCIF factor in human therapy. This SCIF factor may be the human polypeptide encoded by these prior art sequences. The Obaru/Zipfel SCIF sequence is reported below in Table I. Alternatively, this factor may be obtained as described in Example 1 below. The human analog of SCIF was obtained from a human T cell line using oligonucleotides based on the published LD 78

sequence of Obaru et al, cited above. Human SCIF demonstrates human activity identical to the murine protein in the SCIF assays described below.

The human SCIF clone of Example 1 has been substantially sequenced. While exhibiting the required activity, this sequence has been found to differ in both nucleotide and amino acid sequence from the published sequence. The alternative human SCIF DNA and amino acid sequence of Example 1 is reported below in Table II. The sequence within the brackets has not yet been determined. The sequence that does appear within the brackets on Table II has been derived from the published LD 78 sequence of Obaru et al and may or may not be identical with the human SCIF clone. The differences in the nucleotide sequence between the cloned SCIF sequence of Example 1 and the published Obaru sequence are indicated in Table II by asterisks above the changed or added amino acids and below the changed nucleotides. Dashes indicate non-coding sequences not found in the sequence of Example 1. There are 18 nucleotide differences and 4 amino acid differences between the two SCIF sequences. Additionally the human clone includes 3 more nucleotides and an additional amino acid in contrast to the published sequence.

However, also useful in the present invention as a SCIF molecule are other proteins encoded by other DNA sequences, not identical to that of the prior art references. Such DNA sequences are characterized as

5 capable of hybridizing under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the human SCIF (or Obaru et al) DNA sequences and coding on expression for polypeptides or

10 proteins capable of demonstrating SCIF activity as described herein. An example of one such stringent hybridization condition is hybridization at 4XSSC at 65°C, followed by a washing in 0.1XSSC at 65°C for an hour. Alternatively an exemplary stringent

15 hybridization condition is in 50% formamide, 4XSSC at 42°C.

Other human SCIF proteins or polypeptides may be encoded by other DNA sequences which hybridize to the sequences for human SCIF (Obaru et al sequence and the

20 sequence obtained from the clone of Example 1) under relaxed hybridization conditions and which code on expression for peptides having SCIF biological properties. Examples of such non-stringent hybridization conditions are 4XSSC at 50°C or hybridization with 30-40%

25 formamide at 42°C. For example, the molecule identified previously as human MIP-beta, may be useful in the method

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of this invention, if capable of displaying stem cell inhibitory activity as defined hereinbelow by assay.

In a like manner other SCIF polypeptides may be characterized by amino acid sequences which differ from  
5 the Obaru et al sequence or the sequence obtained from the clone of Example 1 due to allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change). SCIF polypeptides encoded by DNA sequences  
10 which differ in codon sequence due to the degeneracies of the genetic code or allelic variations are also expected to be useful in the method of this invention. Variations in the DNA sequence of SCIF which are caused by point mutations or by induced modifications to enhance the  
15 activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention and expected to be useful in the disclosed method.

The method of the present invention may also employ the murine factor (also known as macrophage  
20 inflammatory protein I-alpha, MIP-1 alpha). Murine MIP-1 beta does not have stem cell inhibitory activity. The sequence of the molecule here identified as murine SCIF is reported in Davatelis et al, J. Exp. Med., 167:1939-1944 (1988) incorporated herein by reference. The stem  
25 cell inhibitory function of this molecule was not

recognized in that reference. The murine factor may also be employed in the methods of this invention, provided that it provokes no antibody generation from the human immune system.

5                    Obviously other species analogs of SCIF may be employed in various veterinary uses of the above identified methods.

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TABLE I

AAGGACACGG GCAGCAGACA GTGGTCATGC CTTTCTTGGC	40
TCTGCTGACA CTCGAGCCCA CATTCCGTCA CCTGCTCAGA ATC	83
ATG CAG GTC TCC ACT GCT GCC CTT GCT GTC CTC CTC TGC Met Gln Val Ser Thr Ala Ala Leu Ala Val Leu Leu Cys 1 5 10	122
ACC ATG GCT CTC TGC AAC CAG TTC TCT GCA TCA CTT GCT Thr Met Ala Leu Cys Asn Gln Phe Ser Ala Ser Leu Ala 15 20 25	161
GCT GAC ACG CCG ACC GCC TGC TGC TTC AGC TAC ACC TCC Ala Asp Thr Pro Thr Ala Cys Cys Phe Ser Tyr Thr Ser 30 35	200
CGG CAG ATT CCA CAG AAT TTC ATA GCT GAC TAC TTT GAG Arg Gln Ile Pro Gln Asn Phe Ile Ala Asp Tyr Phe Glu 40 45 50	239
ACG AGC AGC CAG TGC TCC AAG CCG GGT GTC ATC TTC CTA Thr Ser Ser Gln Cys Ser Lys Pro Gly Val Ile Phe Leu 55 60 65	278
ACC AAG CGA AGC CGG CAG GTC TGT GCT GAC CCC AGT GAG Thr Lys Arg Ser Arg Gln Val Cys Ala Asp Pro Ser Glu 70 75	317
GAG TGG GTC CAG AAA TAT GTC AGC GAC CTA GAG CTG AGT Glu Trp Val Gln Lys Tyr Val Ser Asp Leu Glu Leu Ser 80 85 90	356
GCC TGA GGGGTCCAGA AGCTTCGAGG CCCAGCGACC Ala End	392
TCGGTGGGCC AGTGGGGAGG AGCAGGAGCC TGAGCCTTGG GAAACATGCG	442
TGTGACCTCC ACAGCTACCT CTTCTATGGA CTGGTTGTTG CCAAACAGCC	492

ACACTGRGGG ACTCTTCTTA ACTTAAATTT TAATTTATTT ATTTATACTA 542  
TTTAGTTTTT GTAATTTATT TTCGATTCA CAGTGTGTTT GTGATTGTTT 592  
GCTCTGAGAG TTCCCCTGTC CCCTCCCCCT TCCCTCACAC CGCGTCTGGT 642  
GACAACCGAG TGGCTGTCAT CAGCCTGTGT AGGCAGTCAT GGCACCAAAG 692  
CCACCAGACT GACAAATGTG TATCGGATGC TTTTGTTTCAG GGCTGTGATC 742  
GGCCTGGGGA AATAATAAAG ACGCTCTTTT AAAAGGTAAA AAAAAAAAAA 792  
AAAAAA 798

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TABLE II

[illegible]

21

ACTTAAATTT TAATTTATTT ATTTATACTA TTTAGTTTTT GTAATTTATT 515  
TTCGATTTC AAGTGTGTTT GTGATTGTTT GCTCTGAGAG TTCCCCTGTC 565  
CCCTCCCCCT TCCCTCACAC CGCGTCTGGT GACAACC]GA GTGGCTGTCA 615  
TCGGCCTGTG TAGGCAGTCA TGGCACCAAA GCCACCAGAC TGACAAATGT 665  
\*  
GTATCAGATG CTTTTGTTCA GGGCTGTGAT CGGCCTGGGG AAATAATAAA 715  
\*  
GATGTTCTTT TAAACGGTAA AAA----- ----- 738  
\* \* \*

This stem cell inhibitory function of LD78 or murine MIP alpha identified in the references described above, has heretofore never been identified as a function of this protein despite the extensive work of researchers on this factor. See, e.g., Obaru et al and Zipfel et al, cited above.

The stem cell inhibitory factor acts on cycling stem cells by reversibly placing them in an undividing "resting" state. When the stem cells are initially treated in this way, the subsequent application of chemotherapy or radiation does not kill the stem cells because the cells are not dividing. Thus, after exposure to the chemotherapy or radiation, the stem cells may be "reactivated" to generate dividing progenitor cells, by discontinuing the administration of SCIF.

An additional means of stimulating the resting stem cells into division may also be the administration to the subject of other colony stimulating factors, e.g., M-CSF, CSF-1, GM-CSF, G-CSF, Meg-CSF or other cytokines, such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11 and erythropoietin following the chemotherapy or radiation exposure.

SCIF polypeptides or active fragments having stem cell inhibitory activity, may be produced by known conventional chemical synthesis or recombinant techniques employing the amino acid and DNA sequences of Table I or Table II, above. For example, SCIF polypeptides may be produced by culturing a suitable cell or cell line, which has been transformed with a DNA sequence coding on expression for a SCIF polypeptide or active fragment thereof under the control of known regulatory sequences. The resulting protein may be isolated from the cells, cell lysate, or medium by conventional techniques. Suitable techniques for such production of recombinant SCIF are described in, e.g., T. Maniatis et al, Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

Methods for constructing SCIF polypeptides useful in the method of the present invention by chemical synthetic means are also known to those of skill in the art. See, e.g., Merrifield in J.A.C.S, 85: 2149-2154 (1963) or "Peptide Synthesis" by Bodanszky, et al, second edition, John Wiley and Sons, 1976.

The recombinant or synthetically-constructed SCIF polypeptide sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with SCIF polypeptides may possess the

same biological characteristic of inhibiting stem cell division in common with the human factor identified above in Table I or Table II.

Modifications in the peptides or DNA sequences encoding SCIF or active fragments thereof may also be made and are believed to be useful also in the method of this invention, where the modified SCIF peptide or fragment thereof shares the desired stem cell inhibitory biological activity. Modifications of interest in the SCIF sequences may include the replacement, insertion or deletion of a selected amino acid residue in the coding sequences. Mutagenic techniques for such replacement, insertion or deletion are well known to one skilled in the art. [See, e.g., United States Patent No. 4,518,584.]

Specific mutations of the sequences of the SCIF polypeptide may involve the insertion of an asparagine-linked glycosylation site, either Asp-X-Thr or Asp-X-Ser, where X can be any amino acid, into the sequence or a sequence modification at any site of the molecule that is modified by addition of O-linked carbohydrate.

Other analogs and derivatives of the sequence of SCIF which would be expected to retain SCIF activity in whole or in part may also be easily made by one of skill in the art and may be useful in the methods of this invention.

Recombinant production of SCIF is presently preferred. The human cDNA may be isolated and inserted into suitable cells or cell lines under the control of appropriate regulatory sequences. Suitable host cells for production of the protein may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U. S. Patent No. 4,419,446. Other suitable mammalian cell lines are the monkey COS-1 cell line, and the CV-1 cell line.

Similarly useful as host cells suitable for the production of SCIF for use in the present invention are bacterial cells. For example, the various strains of E. coli (e.g., HB101, MC1061 and strains used in the following examples) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g., Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

It may also be possible to employ one or more peptide fragments of SCIF, which retain the stem cell inhibitory activity of intact SCIF molecule, for use in the present methods for protecting a subject's stem cells from destruction by exposure to an agent capable of killing dividing stem cells.

For use in the method for protecting stem cells according to this invention a therapeutically effective amount of the SCIF protein or a therapeutically effective fragment thereof may be employed in admixture with a pharmaceutically acceptable carrier. Where desirable, this SCIF composition can be systemically administered parenterally. In clinical applications, it may be desirable to target the SCIF to the blood-forming tissue, e.g., bone marrow. This targeting can be achieved by injecting the SCIF, normally by infusion or bolus intravenous administration. Alternatively, the SCIF can be targeted by varying the pharmaceutical formulation of the drug, e.g. by linking it to agents which have been

shown to target to the bone marrow. This formulation can be administered intravenously. If desirable, the composition may be administered subcutaneously.

When systematically administered, the  
5 therapeutic composition for use in this invention is in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such a pharmaceutically acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is  
10 within the skill of the art. For administration in the method for treating hyperproliferating stem cells, the composition containing SCIF may be administered topically or through a transdermal patch to localize its effect on the area of hyperproliferation.

15 The dosage regimen involved in a method for treating the subject anticipating exposure to such cytotoxic agents or for treatment of hyperproliferating stem cells will be determined by the attending physician considering various factors which modify the action of  
20 drugs, e.g. the condition, body weight, sex and diet of the patient, the severity of any infection, time of administration and other clinical factors. Generally, the daily regimen should be in the range of 1-1000 micrograms of SCIF protein or fragment thereof per  
25 kilogram of body weight.

Following the subject's exposure to the cytotoxic agent or radiation, the therapeutic method of the present invention may also employ administering to the subject one or more lymphokines, colony stimulating factors or other cytokines, hematopoietins, interleukins, growth factors to generally stimulate the growth and division of the stem cells inhibited by the prior treatment with SCIF. Such therapeutic agents which encourage hematopoiesis include IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, Meg-CSF, M-CSF, CSF-1, GM-CSF, G-CSF or erythropoietin. The dosages of these agents may be employed in the same ranges as the dosages for SCIF recited above. In a like manner, these dosages would be adjusted to compensate for variations in the physical condition of the patient, and the amount and type of chemotherapeutic agent or radiation to which the subject was exposed. Progress of the reversal of the inhibition of the stem cells caused by administration of SCIF in the treated patient can be monitored by conventional methods.

In the treatment of leukemia, it may prove beneficial to administer both SCIF to inhibit normal stem cell cycling and a stimulator of leukemic cell growth, such as IL-3 or GM-CSF, simultaneously with the cytotoxic drug treatment or during radiation. By this protocol, it should be possible to achieve the greatest differences

between the cycling statuses of normal and leukemic cells.

The following examples illustratively describe the use of murine SCIF in the in vitro stem cell assay.

5 These examples are for illustration and do not limit the scope of the present invention.

Example 1 - Obtaining Human SCIF cDNA

Polyadenylated RNA isolated from the human T cell line C10-MJ2 was used as a template for synthesis of single stranded complementary DNA by standard procedures [see, e.g., Maniatis et al., cited above]. The presence of cDNA's encoding human SCIF were demonstrated by the polymerase chain reaction (PCR) employing stringent conditions [R. K. Saiki et al., Science, 230:1350 (1985)]  
10 using the following oligonucleotides based on the sequence of Table I:

5' AGCTCGAGAT CATGCAGGTC TCCACTG 3'

5' GCGAATTCCC TCAGGCACTC AGCTCCA 3'.

Double-stranded SCIF DNA obtained as the product of the PCR and labeled with (alpha <sup>32</sup>P) dCTP by random priming was used to identify SCIF cDNAs in a previously constructed C10-MJ2 cDNA expression library  
20 [J. F. Moreau et al, Nature, 336:690 (1988)].

Conditioned media obtained from COS cells transfected with full-length SCIF cDNAs in the proper orientation were analyzed according to the assay described in Example 3. Sequencing of selected clones, named pXMT2.A1, pXMT2.A3 through pXMT2.A9, is ongoing.

The human SCIF sequence of Table II differs substantially from the Obaru et al LD 78 sequence of Table I, from which the oligonucleotides were derived. There are 18 base changes, 4 amino acid changes as well as three additional bases and an additional amino acid.

#### Example 2 - In Vitro Stem Cell Assay

For the detection of stem cells in vitro  $10^4$  murine bone marrow cells from the murine macrophage cell line, J774.2 [P. Ralph et al, J. Immunol., 114:898-905 (1975)] in 4 ml supplemented alpha-modified minimal essential medium (MEM) containing 25% fetal calf or horse serum and 0.3% agar were seeded on top of an underlayer of the same medium containing 0.6% agar, 10% L929 cell conditioned medium (L929 CM, a source of the growth factor CSF-1) and 10% AF1-19T cell conditioned medium (AF1-19T CM) (a source of the growth factor GM-CSF and other uncharacterized stem cell growth factors) in a 6 cm petri dish. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO<sub>2</sub>, 5% O<sub>2</sub>, 85% N<sub>2</sub> for 11 days. Colonies can be stained with INT 2-(p-iodophenyl)-

3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride hydrate overnight.

While some colonies are present in the CFU-A assay having a diameter less than 2 mm, this value was selected as a useful cut-off point after preliminary experiments were performed using cytosine arabinoside. Within individual dishes, colonies with a diameter <2 mm were mainly derived from cells in cycle, whereas colonies >2 mm were found to be derived from minimally proliferating cells. Only colonies with diameters >2 mm were scored in these assays.

The assay, also described in I. B. Pragnell et al, Blood, 72:196-201 (1988), demonstrates that more mature progenitor cells are unaffected by treatment with SCIF, while cycling stem cells, after treatment with SCIF, become resistant to the action of cytosine arabinoside. If the treated stem cells are then washed with buffered saline to remove the cytotoxic drug and SCIF the surviving stem cells proliferate in culture normally as illustrated below.

#### Example 3 - Demonstration of the Effect of SCIF on CFU-A

Bone marrow cells were incubated in paired tubes containing  $5 \times 10^6$  cells in 1 ml Fischer's medium supplemented with 20% horse serum. SCIF or alpha-MEM was added to each tube and Fischer's medium was added to

control tubes. The mixtures were incubated at 37°C for 5 hours (inhibition assays). For the last 60 minutes of the incubation  $10^{-3}$ M cytosine arabinoside was added to one tube and an equal volume of medium to the other tube.

5 Cells were then washed twice before being assayed in the CFU-A assay as described above. SCIF was found to reduce the number of stem cells in cycle from an average of greater than 30% to an average of less than 10%. The untreated cells were killed by the cytotoxic drug  
10 treatment in contrast to the inhibitor treated stem cells.

Pure preparations of SCIF also reversibly trigger multipotential stem cells out of cycle when assayed in vivo using ara-C in the CFU-S assay described  
15 in Pragnell et al, cited above.

The purified and sequenced SCIF of this invention is a cytokine which has the ability to specifically reduce the proportion of haemopoietic stem cells in DNA synthesis, thereby protecting them from  
20 cytosine arabinoside, a cell-cycle specific cytotoxic drug. In contrast, SCIF does not affect the proliferation of more mature progenitors and so appears to be a specific regulator of the stem cell compartment. SCIF is active in the 200-500 pM range, as measured in  
25 the CFU-A direct addition assays.

Example 4 - Expression of Recombinant Human SCIF

To produce SCIF, the cDNA encoding it is transferred into an appropriate expression vector of which numerous types are known in the art for mammalian, insect, yeast, fungal and bacterial expression by standard molecular biology techniques. See, e.g., Y. C. Yang et al, Cell, 47:3-10 (1986). One such vector is the COS cell expression vector, pXM, containing the SV40 enhancer, major adenovirus late promoter, DHFR coding sequence, SV40 late message poly A addition site and VaI gene. This vector may be linearized with the endonuclease enzyme XhoI and ligated to equimolar amounts of SCIF cDNA which has been previously modified by the addition of synthetic oligonucleotides that generate complementary XhoI cohesive ends. Such oligonucleotides are commercially available [Collaborative Research, Lexington, MA]. The vector is then introduced into appropriate host cells by conventional genetic engineering techniques.

20           a.   Mammalian Cell Expression

To obtain expression of the SCIF polypeptide for use in the assays described below, the pXM vector containing the SCIF DNA sequence is transfected onto COS cells, for example. The conditioned medium for the transfected COS cells contains SCIF

biological activity as measured in the assay described in Example 2.

The mammalian cell expression vectors described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors, e.g. replicons, selection genes, enhancers, promoters, and the like, may be obtained from natural sources or synthesized by known procedures. See, Kaufman et al, J. Mol. Biol., 159:511-521 (1982); and Kaufman, Proc. Natl. Acad. Sci., USA, 82:689-693 (1985). Exemplary mammalian host cells include particularly primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable. Candidate cells need not be genotypically deficient in the selection gene so long as the selection gene is dominantly acting. For stable integration of the vector DNA, and for subsequent amplification of the integrated vector DNA, both by conventional methods, CHO cells may be employed. Alternatively, the vector DNA may include all or part of the bovine papilloma virus genome [Lusky et al, Cell, 36:391-401 (1984)] and be carried in cell lines such as C127 mouse cells as a stable episomal element. Other suitable mammalian cell lines include but are not limited to, HeLa, COS-1 monkey cells, mouse L-929

cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Stable transformants are then screened for expression of the product by standard immunological or enzymatic assays. The presence of the DNA encoding the SCIF polypeptides may be detected by standard procedures such as Southern blotting. Transient expression of the DNA encoding the polypeptides during the several days after introduction of the expression vector DNA into suitable host cells, such as COS-1 monkey cells, is measured without selection by activity or immunologic assay of the proteins in the culture medium.

One skilled in the art can also construct other mammalian expression vectors comparable to the pXM/SCIF vector by, e.g., inserting the DNA sequence of SCIF from the respective plasmids with XhoI and employing well-known recombinant genetic engineering techniques and other known vectors, such as pJL3 and pJL4 [Gough et al., EMBO J., 4:645-653 (1985)] and pMT2 (starting with pMT2-VWF, ATCC #67122; see PCT application PCT/US87/00033). The transformation of these vectors into appropriate host cells can result in expression of the SCIF polypeptides.

b. Bacterial Expression Systems

Similarly, one skilled in the art could manipulate the sequence of SCIF by eliminating any mammalian regulatory sequences flanking the coding

sequences and inserting bacterial sequences to create bacterial vectors for intracellular or extracellular expression of the SCIF polypeptides of the invention by bacterial cells. The DNA encoding the factor may be  
5 further modified to contain different codons for bacterial expression as is known in the art. Preferably the sequence is operatively linked in-frame to a nucleotide sequence encoding a secretory leader polypeptide permitting bacterial expression, secretion  
10 and processing of the mature variant protein, also as is known in the art. The compounds expressed in bacterial host cells may then be recovered, purified, and/or characterized with respect to physiochemical, biochemical and/or clinical parameters, all by known methods.

15 c. Insect or Yeast Cell Expression

Similar manipulations can be performed for the construction of an insect vector [See, e.g., procedures described in published European patent application 155,476] for expression in insect cells. A  
20 yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the proteins of the present invention by yeast cells. [See, e.g., procedures described in published PCT application WO 86/00639 and European patent  
25 application EP 123,289.]

Example 5 - Construction of CHO Cell Lines Expressing High Levels of SCIF

One method for producing high levels of the SCIF polypeptides of the invention from mammalian cells involves the construction of cells containing multiple copies of the heterologous SCIF gene. The heterologous gene can be linked to an amplifiable marker, e.g., the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman & Sharp, J. Mol. Biol., (1982) supra. This approach can be employed with a number of different cell types.

For example, the pXM vector containing a SCIF gene in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdd26SV(A)3 (Kaufman & Sharp, Mol. Cell Biol., 3(9):1598-1608 (1983) can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by calcium phosphate coprecipitation and transfection. Alternatively, the SCIF gene may be introduced into pMT2 as previously mentioned and the resultant vector used in place of pXM/SCIF and pAdd26SV(A)3. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of

MTX (sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as described in Kaufman et al., Mol. Cell Biol., 5:1750 (1983). Transformants are cloned, and biologically active SCIF polypeptide expression is monitored by the assay of Example 2. SCIF polypeptide expression is expected to increase with increasing levels of MTX resistance.

Numerous modifications and variations in practice of this invention are expected to occur to those skilled in the art.

CLAIMS

1. A stem cell inhibitory protein substantially free from other human proteinaceous materials and comprising the amino acid sequence of Table II.
  2. A pharmaceutical composition useful for inhibiting stem cell division in a human subject exposed to or anticipating exposure to an agent capable of damaging or destroying stem cells undergoing division comprising an effective amount of a human stem cell inhibitory factor in admixture with a suitable carrier.
  3. The composition according to claim 2 wherein said stem cell inhibitor is all or a biologically active fragment of the human protein having the same or substantially the same amino acid sequence of Table I, an allelic variant thereof, a polypeptide having a DNA sequence capable of hybridizing to the DNA sequence of Table I and having stem cell inhibiting activity, or a biologically active fragment thereof.
  4. The composition according to claim 2 wherein said stem cell inhibitor is all or a biologically active fragment of the protein having the same or substantially the same amino acid sequence of Table II, an allelic variant thereof, a polypeptide having a DNA sequence
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capable of hybridizing to the DNA sequence of Table II and having stem cell inhibiting activity, or a biologically active fragment thereof.

5. A method for preparing an autologous bone marrow sample for transplantation comprising treating said marrow ex vivo with an effective amount of a human stem cell inhibitory factor substantially free from other human proteinaceous materials and comprising the amino acid sequence of Table II to inhibit stem cell division; and purging said marrow of cancerous cells by administering an effective amount of a chemotherapeutic agent or radiation.
6. A DNA sequence consisting essentially of a DNA coding on expression for a stem cell inhibitory protein substantially capable of inhibiting growth of stem cells in humans, said DNA sequence encoding a peptide sequence encompassing the mature form of the polypeptide of Table II and allelic variations thereof also capable of inhibiting the growth of stem cells in humans.
7. A vector comprising a DNA sequence consisting essentially of a DNA coding on expression for a stem cell inhibitory protein substantially capable of inhibiting growth of stem cells in humans, said DNA sequence encoding a peptide sequence encompassing the mature form

of the polypeptide of Table II and allelic variations thereof in association with regulatory control sequences capable of directing the replication and expression thereof in host cells.

8. A method for producing a stem cell inhibitory protein substantially free from other human proteinaceous materials and comprising the amino acid sequence of Table II comprising culturing a cell transfected with the DNA sequence encoding said protein, said DNA under the control of regulatory control sequences capable of directing the replication and expression thereof in said cell.

9. The use of a stem cell inhibitory protein substantially free from other human proteinaceous materials and comprising the amino acid sequence of Table II in a pharmaceutical composition for the treatment of subjects anticipating exposure to agents which damage dividing stem cells.

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 90/05451

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>5</sup> : C 07 K 13/00, A 61 K 35/28, C 12 N 15/19, A 61 K 37/02																	
<b>II. FIELDS SEARCHED</b> <div style="text-align: right; font-size: small;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border-bottom: 1px solid black; font-size: small;">Classification System</td> <td style="border-bottom: 1px solid black; font-size: small;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC<sup>5</sup></td> <td style="padding: 5px;">C 07 K, A 61 K, C 12 N</td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>5</sup>	C 07 K, A 61 K, C 12 N											
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse; font-size: x-small;"> <tr> <th style="width: 10%;">Category <sup>9</sup></th> <th style="width: 70%;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">           The Journal of Immunology, vol. 142, no. 5, 1 March 1989, The American Association of Immunologists, (US), P.F. Zipfel et al.: "Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors", pages 1582-1590 see page 1582, column 2, lines 4-23, 36-42; page 1583, column 1, lines 6-11; page 1585, figure 3B; page 1586, column 1, lines 8-18; discussion cited in the application         </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-4,6-9</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td style="text-align: center; vertical-align: top; padding: 5px;">5</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">           EP, A, 0317141 (BECTON DICKINSON AND CO.)            24 May 1989            see claims 1,2,5            --         </td> <td style="text-align: center; vertical-align: top; padding: 5px;">5</td> </tr> <tr> <td colspan="3" style="text-align: center; padding: 10px;">./.</td> </tr> </table>			Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	The Journal of Immunology, vol. 142, no. 5, 1 March 1989, The American Association of Immunologists, (US), P.F. Zipfel et al.: "Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors", pages 1582-1590 see page 1582, column 2, lines 4-23, 36-42; page 1583, column 1, lines 6-11; page 1585, figure 3B; page 1586, column 1, lines 8-18; discussion cited in the application	1-4,6-9	Y	--	5	Y	EP, A, 0317141 (BECTON DICKINSON AND CO.) 24 May 1989 see claims 1,2,5 --	5	./.		
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<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p><b>* Special categories of cited documents: <sup>10</sup></b></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>																	
<b>IV. CERTIFICATION</b> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; font-size: small;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; font-size: small;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="text-align: center; padding: 5px;">31st January 1991</td> <td style="text-align: center; padding: 5px;">22.02.91</td> </tr> <tr> <td style="border-bottom: 1px solid black; font-size: small;">International Searching Authority</td> <td style="border-bottom: 1px solid black; font-size: small;">Signature of Authorized Officer</td> </tr> <tr> <td style="text-align: center; padding: 5px;">EUROPEAN PATENT OFFICE</td> <td style="text-align: center; padding: 5px;">miss T. MORTENSEN </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	31st January 1991	22.02.91	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	miss T. MORTENSEN							
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	J. Biochem., vol. 99, no. 3, 1986, (Tokyo, JP), K. Obaru et al.: "A cDNA clone used to study mRNA inducible in human tonsillar lymphocytes by a tumor promoter", pages 885-894 see introduction; page 889, figure 3; discussion cited in the application --	1-4,6-9
P,X	Nucleic Acids Research, vol. 18, no. 11, 11 June 1990, S.G. Irving et al.: "Two inflammatory mediator cytokine genes are closely linked and variably amplified on chromosome 17q", pages 3261-3270 see the whole article  -----	1-4,6-9

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

US 9005451  
SA 41492

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The members are as contained in the European Patent Office EDP file on 15/02/91  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0317141	24-05-89	JP-A- 2002345	08-01-90

EP0 FORM P0479

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